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SHORT COMMUNICATION

Novel NTP Binding Property of Rice Dwarf Phytoreovirus Minor Core Protein P5

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Rice dwarf phytoreovirus (RDV) mRNA synthesized from purified virion has a cap structure, m^7GpppAm—, which suggests the presence of guanylyltransferase activity in the virion. We attempted to identify the enzyme involved in the cap formation by using a nucleoside triphosphate binding assay. Incubation of virion with [α-^{32}P]GTP resulted in labeling of an 89-kDa protein that had not previously been identified in purified virus preparations. Interestingly this protein also covalently bound UTP and ATP, which is not a property of the known guanylyltransferases. RDV particles catalyzed GTP-PP_i, dGTP-PP_i, ATP-PP_i, and UTP-PP_i exchange reactions. In SDS–polyacrylamide gel electrophoresis, the 89-kDa protein comigrated with the S5-coded protein, P5, which had been expressed by a baculovirus vector. Moreover, the labeled 89-kDa protein was precipitated by an antiserum against this recombinant RDV P5. Careful reinvestigation of purified virus particles by SDS–polyacrylamide gel electrophoresis and Western blotting analyses showed that they contained a small amount of P5 (<0.5% of the total protein) within the core. These results may suggest that the minor core protein of RDV, which is coded by S5, is a candidate guanylyltransferase, although the biological significance of its ATP and UTP binding activities remains largely unknown.

Rice dwarf virus (RDV) (*1*), a species of the genus *Phytoreovirus* of the family *Reoviridae* (*2*), has a genome composed of 12 segmented double-stranded RNAs designated S1 to S12 according to increasing order of mobility in polyacrylamide gels. The sequences of each of the 12 segments are known, and all the segments except for S2 and S5 are assigned to structural (S1, S3, S7, S8) and nonstructural (S4, S6, S9–S12) proteins (*3*). All the protein products, except for the S12-encoded proteins, are detected in both RDV-infected plant and RDV-infected insect hosts (*4*). Location of the S2- and S5-coded proteins, which are presumed to constitute the virus outer layer (*5*), remains to be determined.

In animal reoviruses, virus particles are reported to contain several enzymatic activities that are responsible for the synthesis of capped and methylated viral mRNA, which include RNA-dependent RNA polymerase (RDRP), RNA triphosphatase, guanylyltransferase, guanine-7-methyltransferase, and nucleoside-$O^{2'}$-methyltransferase activities (*6*). Among the virion-associated enzymes, RDRP is assigned to one of the viral gene products located in the viral core (*7*). Guanylyltransferase, which is involved in the mRNA capping reaction, is also identified on the basis of its specific binding activity with GTP and its GTP-PP_i exchange activity (*8–12*). RDV virion seems to possess similar enzymatic activities; the terminal structure of RDV mRNA synthesized by virion-associated transcriptase is m^7GpppAm— (*13*), which is identical to that of another phytoreovirus, wound tumor virus (WTV) (*14*). Some of the RDV structural proteins, therefore, are likely to be responsible for mRNA synthesis. Nevertheless, only the activity of the RDV S1-coded protein, P1, has been assigned to an RDV structural core protein; P1 shares sequence homology with known RDRPs (*3*).

In the current study, we have shown that the S5 gene product, P5, is not an outer layer protein as previously assumed but is a minor core protein. Moreover, P5 covalently bound GTP, which suggested that P5 may be guanylyltransferase. However, P5 also had UTP and ATP binding activities, which are considered to be unusual for guanylyltransferase.

RDV-infected rice leaves were homogenized as stated by Suzuki *et al.* (*15*). For clarification, freon (fluorocarbon, Daiflon S-3; Daikin Kogyo Ltd., Osaka) or freon plus carbon tetrachloride was used. The extract was centrifuged through a 20–50% sucrose gradient following a cycle of differential centrifugation. The lower and upper bands containing empty and intact virus particles were recovered. Core particles were purified substantially by the method of Takahashi *et al.* (*16*). Virus particles with an A_{260} of 20 prepared as above were incubated in 0.1 *M* histidine, pH 6.2, 0.8 *M* $MgCl_2$ (a total volume is 80 μl)

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for 5 min and centrifuged at 95,000 rpm for 10 min. After decanting the supernatant, the pellet was resuspended in 80 μl of 0.1 *M* histidine, 10 m*M* $MgCl_2$, pH 6.0, incubated for 5 min at room temperature, and centrifuged again at 95,000 rpm for 10 min. The core-particle-containing pellet was resuspended in 60 μl of distilled water.

GTP-PP_i exchange assay was carried out by the method of Mao and Joklik (*12*). Purified virus particles were incubated at 25° for 30 min in 50 μl (final volume) of 20 m*M* Tris–HCl, pH 8.0, 1.5 m*M* $MnCl_2$, 1 m*M* DTT, 1 m*M* one species of the nucleoside triphosphates, 5 m*M* $MgCl_2$, and 50 μM [^{32}P]PP_i. After addition of 100 μl of 0.1 *M* cold $Na_4P_2O_7$, 5 μl (5 μg) of BSA (10 mg/ml), and 500 μl of cold 10% trichloroacetic acid, the reaction mixture was incubated for 30 min at 4° and then centrifuged at 15,000 rpm for 20 min. We added 100 μl of 25% activated charcoal (Wako Pure Chemical, Tokyo) to the supernatant and centrifuged it at 15,000 rpm for 2 min following incubation at 4° for 15 min. The pellet was washed three times with 500 μl of 5 m*M* HCl and subsequently with 500 μl of cold distilled water. The resultant pellet was resuspended in 500 μl of 50% ethanol–2% NH_4OH, incubated at room temperature for 2 min, and centrifuged at 15,000 rpm for 2 min. The NTP-containing, PP_i-free supernatant was recovered. The procedure was repeated. The first and second supernatants were mixed, lyophilized, and resuspended in 50 μl of distilled water. The eluted materials were spotted onto PEI-cellulose sheets (Polygram Cell 300, Macherey-Nagel, Germany) and developed in 0.5 *M* NH_4HCO_3 for 30 min. The labeled substances were detected by autoradiography.

In order to confirm that RDV particles contained guanylyltransferase, a GTP-PP_i exchange assay was performed. RDV particles catalyzed the GTP-PP_i and dGTP-PP_i exchange reaction, as expected (Fig. 1). Surprisingly, RDV particles also possessed ATP-PP_i and UTP-PP_i (shown by an arrowhead) exchange activities. The UTP-PP_i exchange activity was extremely low, relative to the GTP-PP_i, dGTP-PP_i, and ATP-PP_i exchange activities. GDP supported the GTP-PP_i exchange, although the reaction process is unknown. No other substance including CTP, dCTP, or dTTP catalyzed this exchange with [^{32}P]PP_i.

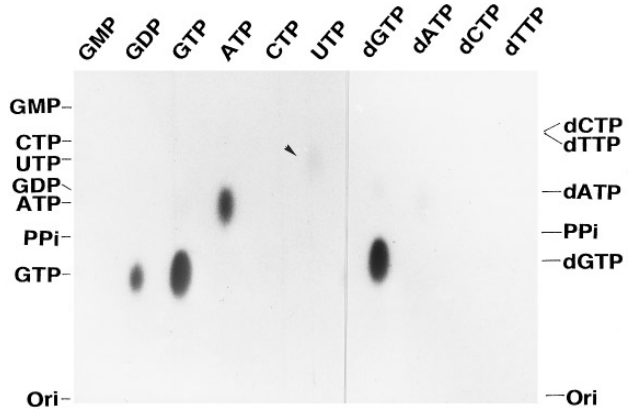

FIG. 1. NTP-PP_i exchange activity of RDV virus particles. [^{32}P]PP_i was added to virus particles in the presence of GMP, GDP, GTP, ATP, CTP, UTP, dGTP, dATP, dCTP, or dTTP (shown on the top) as substrate. The resulting products were separated by thin-layer chromatography after removal of free PP_i using activated charcoal (see text). A radiolabeled, weak signal at the position of UTP is shown by an arrowhead. Standard nucleotides (shown on the right and left) were located under ultraviolet light.

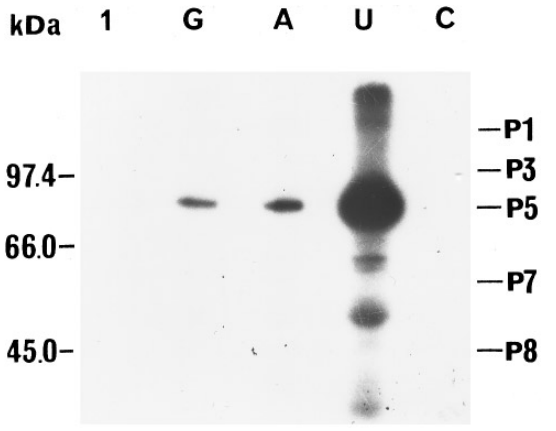

FIG. 2. Mononucleotide binding activity of an RDV structural protein. Twenty micrograms of purified RDV particles was incubated for 2 min in a total volume of 20 μl of 20 m*M* Tris–HCl, pH 8.0, 1 m*M* DTT, 1.5 m*M* $MnCl_2$, and 0.17 μM [α-^{32}P]NTP (3000 Ci/mmol, NEN, MA). Lane G, [α-^{32}P]GTP; lane A, [α-^{32}P]ATP; lane U, [α-^{32}P]UTP; lane C, [α-^{32}P]-CTP. SDS-dissociated RDV proteins were labeled with [α-^{32}P]GTP and processed in the same manner shown above (lane 1). The labeled proteins were subjected to SDS–polyacrylamide gel electrophoresis (*20*) and subsequent autoradiography. Phosphorylase *b* (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), and soybean trypsin inhibitor (20.1 kDa) were used as molecular standards in this and subsequent figures. These standards and the RDV structural proteins were visualized by Coomassie brilliant blue staining before autoradiography.

All earlier reports of guanylyltransferase activities demonstrated covalent binding to GTP. The RDV structural proteins were assayed for NTP binding. As shown in Fig. 2, a polypeptide of 89 kDa in a purified RDV preparation was specifically labeled by [α-^{32}P]ATP, GTP, and UTP (lanes A, G, U). The polypeptide preferentially bound UTP. No band was observed with CTP (lane C), although longer exposure revealed a faint band at the same position (data not shown). The radioactivity remained associated even after boiling in the presence of SDS (2%) and 2-mercaptoethanol (1%). This reaction was detected only when virus particles were not denatured. Dissociation of the virion in 0.1% SDS resulted in loss of the GTP binding capability (Fig. 2, lane 1). This was also true for ATP and UTP binding (data not shown). Taken together, these results showed that the polypeptide and the mononucleotides bound covalently and ruled out the possibility of nonspecific binding during SDS–PAGE. Mg^{2+} could substitute for Mn^{2+} in the binding reaction, while in the absence of divalent cation (Mg^{2+}, Mn^{2+}), no binding activity was observed (data not shown). ATP or GTP did not

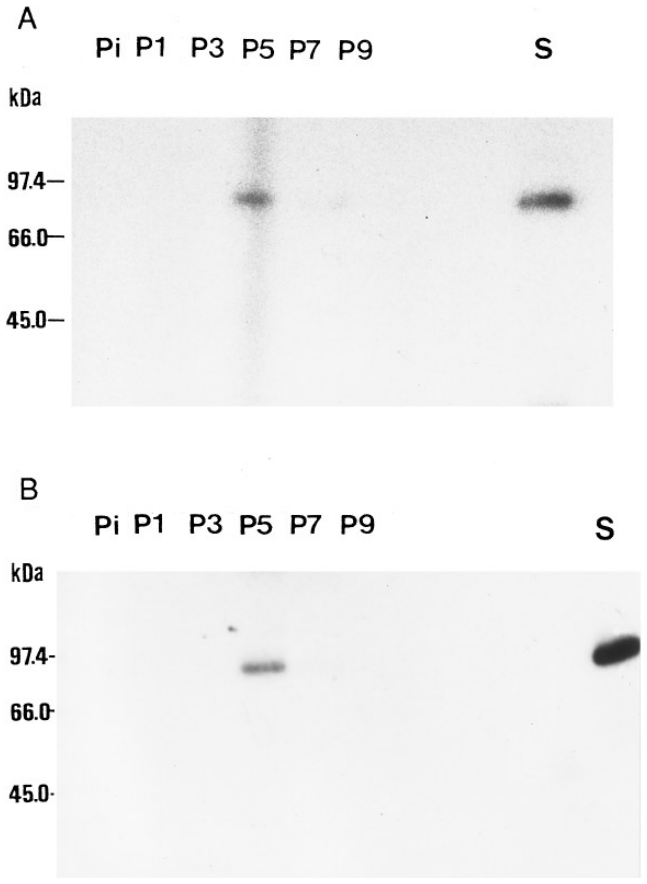

FIG. 3. Immunoprecipitation of a nucleotide binding protein. After treatment with (A) [α-^{32}P]UTP or (B) [α-^{32}P]GTP as stated above, virus particles were denatured by boiling for 1 min in 1× SDS–PAGE sample buffer. 40 μl of the sample was incubated with 2 μl of rabbit antisera against RDV-coded proteins shown on the top (Pi, preimmune rabbit serum; P1, rPAb-P1T; P3, rPAb-P3; P5, rPAb-P5; P7, rPAb-P7; and P9, rPAb-Pns9). The antisera used were those previously described (*4*), except for rPAb-P3, prepared in this study. The immunocomplex was adsorbed to *Staphylococcus aureus* Cowan I cells (IgGsorb, Enzyme Center, U.S.A.). The complex was precipitated by centrifugation at 10,000 rpm for 2 min and washed three times with 250 μl of 150 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris–HCl, pH 7.5, containing 0.05% Triton X-100 (NETT) (*21*). The final pellet was suspended in 40 μl of 1× SDS–PAGE buffer and subjected to SDS–PAGE. Labeled proteins were detected by autoradiography. S refers to the original sample before the immunoprecipitation treatment.

label the protein when the labeled phosphate was in the γ-position (data not shown).

We tried to identify the polypeptide using immunoprecipitation analysis. Antisera were raised against RDV P1 (rPAb-P1T), P3 (rPAb-P3), P5 (rPAb-P5), P7 (rPAb-P7), and Pns9 (rPAb-Pns9), all of which were cloned and produced by recombinant baculoviruses in *Spodoptera frugiperda* cells. Among these only rPAb-P5 precipitated the UTP binding protein (Fig. 3A). Also, the protein labeled by [α-^{32}P]GTP was precipitated with rPAb-P5 (Fig. 3B). This suggested that P5 was a previously unidentified, minor structural protein. We tried to detect P5 in virus particles by Western blotting analysis with rPAb-P5. On Western blot the antibodies bound a polypeptide from core particles and from virion preparations that had a molecular mass of 89 kDa (Fig. 4, WB, lanes 1 and 2). Detection was possible with Coomassie brilliant blue staining (Fig. 4, CBB, lanes 1 and 2), though the amount detected was small, less than 0.5% of the total structural proteins as calculated from densitometric tracing. The mobility of this protein in the gel was the same as that of P5 expressed by a baculovirus recombinant (Fig. 4, WB, lanes 4 and 1).

Seven structural proteins are present in purified RDV preparations from virus-infected rice plants (*5*). Genome segments S1, S3, S7, S8, and S8 are the genes for proteins P1 (170 kDa, minor core), P3 (110 kDa, major core), P7 (58 kDa, minor core), P8 (43 kDa, outer capsid), and P8′ (<39 kDa, outer capsid), respectively (*3*). By analogy to similar WTV proteins, RDV P2 and P5 are assumed to be the outer layer proteins that S2 and S5 encode (*5*). However, we demonstrated that P5 was a minor core protein encoded by S5, not an outer layer protein. That demonstration was based on the following observations: (1) the S5 gene product expressed by a recombinant baculovirus comigrated with P5 in purified virus on an SDS–PAGE gel (Fig. 4, WB, lanes 1 and 4), (2) an antiserum (rPAb-P5) raised against the baculovirus-expressed S5 product specifically bound P5 (Fig. 4, WB), and (3)

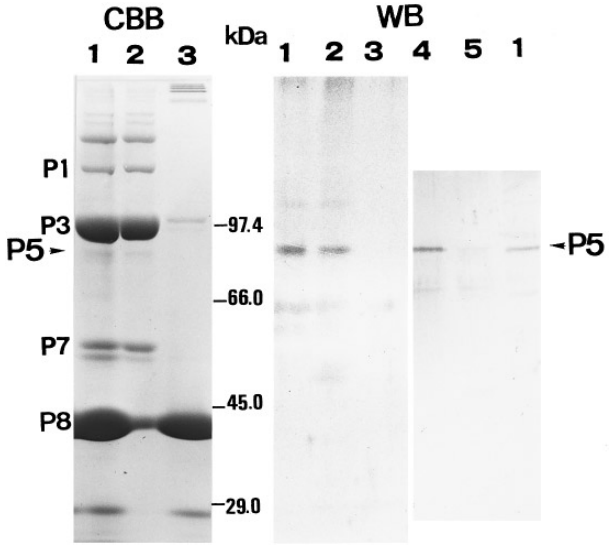

FIG. 4. Detection of RDV P5 in purified virus preparation. Proteins were applied to a 10% (acrylamide:bisacrylamide, 70:1) gel and stained with 0.2% Coomassie brilliant blue R-250 (CBB). Lane 1, purified virus particle; lane 2, purified virus core particle; lane 3, outer capsid removed after core particle purification. Assignment of the RDV structural proteins is shown on the left. Proteins in aliquots of the same samples shown above (lanes 1–3) were electrotransferred (*22*) to a PVDF membrane and immunoenzymatically detected with the aid of an ECL Western blotting kit (Amersham Life Science, Buckinghamshire, England) (WB). Proteins in *S. frugiperda* cells infected with AcRS5 (lane 4) and AcNPV (lane 5) were probed with the anti-RDV P5 rabbit antiserum (rPAb-P5) diluted 500-fold. Apparent molecular masses are 170 kDa for P1, 110 kDa for P3, 89 kDa for P5, 58 kDa for P7, and 43 kDa for P8.

purified core particle preparations contained P5 (Fig. 4, CBB).

We showed that RDV particles had a GTP- and dGTP-PP_i exchange activity like that of other reoviruses, and that a minor core protein, P5, bound nucleoside triphosphates including GTP. Reovirus λ2 (*8, 9, 12*), rotavirus VP3 (*11*), and bluetongue virus VP4 (*10*) are guanylyltransferases (or candidate guanylyltransferases) that bind GTP covalently. RDV P5 had several properties in common with these proteins; it was a core protein that covalently bound GTP and lacked the consensus sequence KXDG, which is commonly found in other guanylyltransferases as well as in DNA and RNA ligases (*17*). RDV P5 differed from the previously reported RNA guanylyltransferases in several ways; it bound not only to GTP and dGTP but also to ATP and UTP, whereas other guanylyltransferases, whether viral or cellular in origin, specifically bind GTP and dGTP. Our results differed from those of Wachsman *et al.* (*18*), who reported that reovirus core particles (probably guanylyltransferase contained in the core) catalyze only the GTP-PP_i exchange reaction when ribonucleoside triphosphate species are tested individually. Also, purified reovirus guanylyltransferase λ2 only catalyzes the GTP-PP_i exchange (*12*). On the contrary, RDV particles had GTP-, UTP-, ATP-, and dGTP-PP_i exchange activities. These differences suggested that RDV P5 is distinct from other reovirus guanylyltransferases.

A discrepancy appeared between the activities evaluated by the two assays (Figs. 1 and 2); P5 primarily bound UTP while the predominant exchange activity of virion preparations was with GTP. The RDV P5 binding activity with UTP and ATP, which was partially associated with the ATP- and UTP-PP_i exchange activity, may also be involved in regulation of another unidentified function of P5, which would not necessarily be part of an efficient exchange reaction with PP_i. It is noteworthy that *Escherichia coli* enzymes involved in glutamine synthesis are regulated by reversible covalent modification with ATP and UTP (*19*). We want to learn whether the nucleotide attachment site of RDV P5 is single or multiple, which amino acid residue(s) constitute(s) the site, and whether the site is shared by GTP, ATP, and UTP. We hope to determine whether UMP and AMP associated with RDV P5 can function as cap donors, and if not, what is their biological significance? Isolation of functionally active RDV P5 from virus particles or from *S. frugiperda* cells infected with RDV S5-carrying recombinant baculovirus, AcRS5, will help answer these questions.

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REFERENCES

1. Iida, T. T., Shinkai, A., and Kimural, I., *In* "CMI/AAB Descriptions of Plant Viruses," No. 102. Commonwealth Mycolog. Inst., Kew, UK, and Assoc. Appl. Biol., 1972.
2. Boccardo, G., and Milne, R. G., *In* "CMI/AAB Descriptions of Plant Viruses," No. 294. Commonwealth Mycol. Inst. Kew, UK, and Assoc. Appl. Biol., 1984.
3. Suzuki, N., *Semin. Virol.* 6, 89–95 (1995).
4. Suzuki, N., Sugawara, M., Kusano, T., Mori, H., and Matsuura, Y., *Virology* 201, 41–48 (1994).
5. Nakata, M., Fukunaga, K., and Suzuki, N., *Ann. Phytopath. Soc. Japan* 44, 288–296 (1978).
6. Schiff, L. A., and Fields, B. N., *In* "Virology" (B. N. Fields, D. M. Knipe, R. M. Charock, M. S., Hirsch, J. L. Melnick, and B. Roizman, Eds.), 2nd ed., pp 1275–1306. Raven Press, New York, 1990.
7. Bruenn, J. A., *Nucleic Acids Res.* 19, 217–226 (1991).
8. Cleveland, D. R., Zarbl, H., and Millward, S., *J. Virol.* 60, 307–311 (1986).
9. Fausnaugh, J., and Shaktin, A. J., *J. Biol. Chem.* 265, 7669–7672 (1990).
10. Le Blois, H., French, T., Mertens, P. P. C., Burroughs, J. N., and Roy, P., *Virology* 189, 757–761 (1992).
11. Liu, M., Mattiopn, N. M., and Estes, M. K., *Virology* 188, 77–84 (1992).
12. Mao, Z., and Joklik, W. K., *Virology* 185, 377–386 (1991).
13. Shimotono, K., and Miura, K., *Annu. Rep. Natl. Inst. Genet.* 26, 13 (1975).
14. Nuss, D. L., and Peterson, A. J., *J. Virol.* 39, 954–957 (1981).
15. Suzuki, N., Watanabe, Y., Kusano, T., and Kitagawa, Y., *Virology* 179, 446–454 (1990).
16. Takahashi, Y., Tomiyama, M., Hibino, H., and Omura, T., *J. Gen. Virol.* 75, 269–275 (1994).
17. Fresco, L., and Buratowski, S., *Proc. Natl. Acad. Sci. USA* 91, 6624–6628 (1994).
18. Wachsman, J. T., Levin, D. H., and Acs, G., *J. Virol.* 6, 563–565 (1970).
19. Bueno, R., Pahel, G., and Magasanik, B., *J. Bacteriol.* 164, 816–822 (1985).
20. Laemmli, U. K., *Nature (London)* 227, 680–685 (1970).
21. Shirako, Y., and Ehara, Y., *J. Gen. Virol.* 67, 1237–1245 (1986).
22. Towbin, H., Staehelin, T., and Gordon, J., *Proc. Natl. Acad. Sci. USA* 76, 4350–4354 (1979).